Precise Measurement of Time Dependent Cell Growth and Detection of Partial Drug Resistance in Infectious Microbial

Qingfeng Hu[2a], Dongshi Gu[1a], Li Xie[3a], Yan Yu[1], Xingrou Chen[1], Jingjing Rui[1], Ning Xu[1], Jennifer Jin Ruan[4], Christopher Dowson[5], Benfang Helen Ruan[1*]

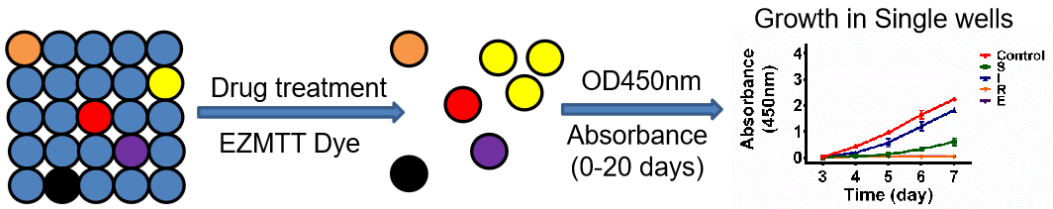
[1]College of Pharmaceutical Science, Collaborative Innovation Center of Yangtza River Delta Region Green Pharmaceuticals, IDD & CB, Zhejiang University of Technology, Hangzhou 310014, China
[2] Clinical Diagnostic lab, Renming Hospital of Zhejiang Province, Hangzhou, 310014, China
[3]Center for M. tuberculosis Research, Hangzhou, 310019 China
[4]Department of Surgery, Memorial Sloan Kettering Cancer Center, NYC, 10009, USA
[5]School of Life Sciences, University of Warwick. CV47AJ, U. K.

***Corresponding author**
Benfang Helen Ruan (Ph. D)
Professor of Pharmaceutical Science
College of Pharmaceutical Science, Collaborative Innovation Center of Yangtza River Delta Region, IDD & CB, Green Pharmaceuticals, Zhejiang University of Technology
E-mail: ruanbf@zjut.edu.cn; ruanbf@yahoo.com
Tel: 86-18357023608
Fax: (0086) 571-88871098
¤Current address: 18 Chaowang Road, Xiachengqu, Hangzhou, Zhejiang, China, 310014

Abstract:
Drug resistance has become a serious public health problem. Accurate assessment of the drug-induced proliferation (DIP) rate is essential in identifying partial drug resistance. The turbidity assay is a continuous assay and has been used for years to track the bacteria growth, but the method has relative low sensitivity and high experimental error. In addition, most current cell proliferation assays are endpoint assays that are not ideal for tracking minor changes over time. Here, we report an EZMTT dye-based detection method which provides 10 to 30-fold better sensitivity than the turbidity assay. Simple mixing of the EZMTT dye with the bacteria in the presence of an inhibitor allowed precise measurement of the drug-induced proliferation rate in high-through-put (HTS) mode; the resulting growth curves show critical characteristics of exponential growth including the cell density requirement and the doubling time. Within 4-6 hours, the simple procedure not only reliably detects all drug resistance found by the traditional method, but also partial drug resistance of infectious bacteria (with 5-20% growth), which current MIC-based clinical diagnostic methods failed to identify. In conclusion, this simple EZMTT method provides rapid and precise measurement of the drug-induced proliferation rate, and identifies 10-30% more partially drug resistant bacteria than current clinical diagnostic methods. A combination of the EZMTT dye-based detection and the DPI method has great potential to solve the unmet medical need to battle against drug resistance.

Keywords. Partial drug resistance, infectious disease, EZMTT, drug-induced proliferation rate, clinical diagnosis, drug discovery

Table of Contents Graphic



Introduction

Bacterial resistance to antibiotics is a serious problem worldwide. It prolongs hospital stays and considerably increases infectious disease-related mortality[1,2]. Research groups[3-6] have demonstrated that drug resistance develops owing to a small population that is resistant to the drug, and drug treatment results in selection for the growth of the small drug-resistant cell population. Therefore, a sensitive method that reliably detects minor growth after drug treatment would be important for the early recognition of drug resistance.

Traditionally, antibiotic susceptibility testing (AST) uses a cell density-based method (**Fig.** 1) for rapidly growing bacteria, such as the turbidity assay, paper diffusion, broth dilution, agar dilution, E-test experimental methods and most recently the automated VITEK drug sensitivity analysis system for clinical diagnosis[7]. The turbidity assay has limited sensitivity, so clinically 80% growth inhibition is used as the cutoff to identify the resistant bacteria. Even though the turbidity assay gives a weak signal, it is used because the assay is simple and continuous.

The indirect impedance method [8] measures the bioelectrical signal changes in response to cell numbers, but requires complicated infrastructure. The BactT/ALERT routine blood culture bottles [9,10] detect microbial growth by oxygen depletion which requires anaerobic conditions, so the bacteria must be grown in a sealed tube or compartment, which is problematic for high-throughput plate-based AST assays. The MTT assay is a traditional method for IC_{50} measurement, but MTT kills the bacteria during the assay [11]. Therefore, as an end point assay, MTT method can not be used to track the drug-induced proliferation rate (DIP) [5].

Therefore, it is critically important to develop a sensitive continuous assay that reliably detects minor growth after drug treatment. Since the EZMTT reagent was shown to have high sensitivity and is capable of tracking time-dependent cell growth[12,13], we further developed the EZMTT-based continuous assays to measure the DIP rate for infectious bacteria. As expected, the assay provided rapid, reproducible and precise assessment of drug-resistance and importantly, reliable detected10-30% more partial drug resistant bacteria than the current turbidity-based diagnostic method..

Results and conclusion:

EZMTT method provides 10-30 times greater sensitivity in cell growth measurement than the traditional turbidity (OD_{600nm}) method

For decades, fast growing infectious bacteria have been tracked by the cell number-based turbidity assay at $OD_{600\ nm}$ which is not very sensitive. As shown in **Fig.** 2, the signal generated by the EZMTT method (**Fig.** 2A-L) is 10-30 times greater than the turbidity assay (**Fig.** 2a-l) for *E. coli* strains (DH5a and TransG1) and 10 clinically isolated infectious bacteria.

Significantly, different *E. coli* strains (**Fig.** 3A-3F; table) demonstrated differences in doubling time and in the required cell density to enter into exponential growth. The infectious species EAEC, EIEC, EPEC and ETEC enter exponential growth at a cell density that is at least four times less than those of DH5a and TransG1. In addition, these infectious species grew approximately two times faster than DH5a and TransG1.

Among other infectious bacteria (**Fig.** 3G-3L), *Bacillus cereus* enters into exponential growth at a relatively low cell density, and the growth rate is rapid enough to have severe consequences after infection. *Salmonella paratyphi A* and *Salmonella* are also fast growing bacteria with doubling times of 0.7-0.8 h.

In addition, the EZMTT method provides highly sensitive detection of both *Staphylococcus aureus* and *Bacillus cereus*. *Staphylococcus aureus* is a common and serious infection associated with high rates of mortality[10], whose growth is only marginally detected by the turbidity assay. The EZMTT assay enhances the detection of growth of both bacterial species by 30-fold, making the drug resistance test more sensitive.

EZMTT provides rapid and precise IC_{50} values for bacterial species

Since the EZMTT dye shows a strong response to the growth of various bacteria (**Fig. 2**), we tried a 4-hr AST of the bacteria using ampicilin (AMP), kanamycin (KAN), and gentamincin (GEN). As shown in **Fig. 3**, the EZMTT-dye based AST showed high assay reliability with an excellent z factor of 0.7-0.8, and provided precise IC_{50} measurements in 4 hrs

for *Staphylococcus aureus* and *Bacillus cereus*, for which the turbidity-based assay requires overnight culture. All the tested strains were sensitive to KAN and GEN. AMP is a potent inhibitor for *Staphylococcus*, but not for *E. coli* and *Bacillus cereus*.

EZMTT demonstrates that changes in IC_{50} value at different cell densities and incubation times are related to mechanism of action

To evaluate the effect of cell-density or incubation time on AST, we measured IC_{50} values of 8 antibiotics in the EAEC *E. coli* strain. IC_{50} values shown in Table 1 and **Fig.** 4 demonstrate the correlation between the mechanism of drug action and the cell-number or incubation time dependent IC_{50} changes.

The antibiotic KAN[14] "irreversibly" binds to 30S-subunit proteins and 16S rRNA, while RIF[15] acts via the inhibition of DNA-dependent RNA polymerase. Both inhibited EAEC cells with essentially the same IC_{50} values regardless the difference in cell density or incubation time; perhaps a suppression of RNA or protein synthesis leads to immediate inhibition of cell growth and causes cell death.

NIT[16] requires initial activation by bacterial flavoproteins, and the activated nitrofurantoin can then either inhibit ribosomal proteins or damage DNA, RNA, protein, and cell wall synthesis. Interestingly, the potency of NIT weakened with prolonged incubation time (24 hr versus 4 hr), perhaps because at the stagnation period of growth at 24h, NIT was not activated by the bacterial flavoproteins.

AMP[17] inhibits penicillin-binding proteins (PBPs) located inside the bacterial cell wall. EAEC was resistant to both AMP and PP, but was sensitive to other penicillin-binding protein inhibitors (CAZ, FEP and FOX). Interestingly, FOX showed essentially the same IC_{50} at different the cell density or incubation time, but CAZ and FEP showed more than 10-fold higher potency at low cell density; the possible explanation is that both CAZ and FEP have multiple cellular targets such as penicillin-binding proteins and beta-lactamase.

EZMTT-based DIP rate measurement identifies 10-30% more partial drug resistance than the traditional turbidity-based clinical diagnostic methods

As shown in Table 1, AST testing at low cell density (400 dilutions from 0.5 MFC) sometimes gives smaller IC_{50} values than those tested at higher cell density (40 dilutions from 0.5 MFC), but after 24 hrs incubation time, the IC_{50} values are similar for most antibiotics. Also, the clinical diagnostic VITEK method uses a 20-fold dilution of a 0.5 MFC bacteria cultures. Accordingly, we evaluated the antibiotic resistance of the 6 clinically isolated *E coli* strains (labeled as EAEC, EIEC, EPEC, ETEC, A) using a 40-fold dilution of a 0.5 MFC bacterial cultures (Table 2, **Fig. 5** and Table 3).

As shown in Table 2, the inhibitory activity of the 30S ribosomal inhibitors (AMK, GEN, MNO and NIT) weakened with prolonged incubation time; perhaps because early phase growth is more dependent on protein synthesis. In contrast, bacterial cell wall synthesis inhibitors such as CSL, CZO, CTX, FEP, TZP, CXM, CAZ, FOX, MEM gained potency with time, and showed reduced IC_{50} values after 24h treatment; perhaps because inhibition of bacterial cell wall synthesis has a late effect on cell growth.

Current clinical AST methods measure MIC or IC_{50} values at 24 hrs after drug treatment and use 80% inhibition as the cut-off. According to that criterion, 11 out of 75 tests listed in Table 2 were identified as drug resistant. However, when we plotted the time-dependent growth after antibiotic treatment (**Fig. 5**), the DIP rates of the highest antibiotic concentration were calculated and presented in Table 3. Because the DIP rate of no-drug treated test was 0.12-0.13 OD/hrs, we selected the slope of 0.012 OD/hrs as the 90% inhibition cut-off. Using 90% as the threshold, 15 more tests were identified as partial inhibitors with a slope above 0.012 OD/hrs, which corresponds to 10-20% growth in comparison with the no-drug control (DIP rate is approximately 0.12 OD/hrs; 100% growth).

Further, the EZMTT- based AST results for *E. coli* strains were compared with the clinical testing results. As shown in Table 3, using the 90% inhibition as the cut-off, the EZMTT method identifies more drug resistance in clinically isolated *E. coli* strains than the current diagnostic methods; EZMTT method identified all resistant or partially resistant bacteria that were determined by the clinical methods, and in addition, determined10-30% more partial inhibitors that were not detected in the clinical turbidity-based VITEK or KB methods.

Conclusion:

In vitro cell proliferation assays are important tools for drug discovery and clinical diagnosis. Theoretical modeling and experimentation demonstrated that simple IC_{50} measurement is not precise enough to identify partial drug resistance, and the DIP rate method was proposed as an unbiased metric to measure antiproliferative drug efficacy[18]. Because the DIP rate measurement requires time-dependent growth data, a continuous assay is preferred, because the continuous assay can easily collect data from the same sample, whereas the end point assays had to collect data from different samples.

The turbidity assay is a continuous assay and is preferred by the VITEK method to determine antibiotic resistance by the DIP-based method. However, owing to the low sensitivity of the turbidity assay, the VITEK requires longer assay times, and the MIC cut-off is set at 20%.

EZMTT provides a continuous cell viability assay with 10-30 fold enhancement in sensitivity over the turbidity assay. Therefore, the EZMTT-based assay can be used in the DIP method and reliably detect the bacterial growth as low as 5-20%.

Applying the EZMTT method to various clinically isolated infectious bacteria, we obtained reproducible IC_{50} values within 4 hrs. In addition, the assay is sensitive enough to reliably detect IC_{50} changes in response to different incubation times and/or cell densities. The most important application of the EZMTT-dye is to reveal 10-30% more cases of partial drug resistance (5-10% growth) that escaped detection under current clinical “radar”. Application of the EZMTT-based AST method will allow doctors to obtain precise resistance information on a large panel of antibiotics within 4-6 hours and to prescribe the most efficacious medicine to patients; this will avoid prolonged medical treatment and generation of more antibiotic resistant bacteria. Therefore, if implemented with VITEK type automation technology, the EZMTT-based AST method is expected to be a promising tool in combating the worldwide crises of multi-drug resistance in infectious diseases and cancer.

Materials and methods

Materials

Mueller-Hinton Broth was purchased from OXOID（Hampshire，U.K；Chemicals from Sigma (USA), EZMTT purchased from JNF Bioscience (China; USA), CCK-8 or WST-8 from Beyotime Biotechnology (China), *Enterococcus casseliflavus* Collins et al. ATCC700327, *Escherichia coli* (Migula) Castellani and Chalmers ATCC 25922，*Pseudomonas aeruginosa* (Schroeter) Migula ATCC 27853，*Staphylococcus aureus subsp.* aureus Rosenbach ATCC 25923 were purchased from ATCC (USA). Pathogenic *Escherichia coli* (EPEC), invasive *E.coli* (EIEC), toxigenic *E.coli* (ETEC), adherent *E. coli* (EAEC), *Shigella sonnei*, *Shigella flexneri*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella paratyphi A* were isolated from clinical samples and characterized based on Diagnostic Criteria and Principles of Management (WS271-2007, WST287-2800, WS/T80-1996, WS280-2008, WS271-2007)[18-21]. Antibiotics such as Amikacin (AMK), Cefoperazone/Sulbactam (CSL), Cefazolin (CZO), Cefotaxime (CTX), Cefepime (FEP), Imipenem (IPM), Ciprofloxacin (CIP), Sulfamethoxazole (SXT), Gentamicin (GEN), Piperacillin/tazobactam (TZP), Cefuroxime (CXM), Ceftazidime (CAZ), Cefoxitin (FOX), Meropenem (MEM), Minocycline (MNO), Nitrofurantoin (NIT),Ampicillin (AMP), Kanamycin (KAN), Piperacillin (PP), Rifampicin (RIF) purchased from Solarbio LLC (Beijing, China) or Kangtai LLC (Wenzhou, China). The antibiotic mechanism of actions are found at www.db.yaozhi.com. Drug containing cassetts (AST-GNS16) were used to measure the

antibiotic resistance in *E. coli* and read by VITEK 2 compact automatic microbiology instrument from Merieux LLC (France) .

Clinical drug resistant diagnostic method for *Escherichia coli*

Various *E. coli* strains were isolated from clinical samples and plated on MH plate according to standard procedure[22]. Colonies (3-5 each) from the overnight culture plate were diluted in 0.45% NaCl buffer (pH 4.5～7.2) to make a solution with 0.5 MCF turberdity. Then, a 20-fold dilution was made and dispensed into the AST-GNS16 card (*E. coli* drug-resistant testing card) for automatic reading by the VITEK 2 compac system to obtain drug resistant information.

EZMTT method for growth curves and doubling time measurement

Freshly cultured cells were used for growth curve measurement. For gram positive or negative bacteria (e.g. *E. coli*), cells were resuspended in MH broth to prepare r a 0.5 MCF solution. Then, two-fold bacteria dilutions (0-0.5 MCF) were made using the MH broth containing 1X EZMTT. The cells were grown at 37 ˚C and tracked every 1 hour for 24 hours. For comparison, cultures without 1X EZMTT were carried out as controls under the same condition, and aliquots (100 μl) were taken for cell number measurement by colony formation. The doubling times were calculated by linear curve fitting the log phase data points (absorbance at 450 nm & growth time) to obtain the linearity equation, which was then used to calculate the time needed to reach the same absorbance (e.g. $OD_{450\ nm}$=1). The average time needed for each two-fold dilutions to reach the same absorbance at the log phase is recorded as the doubling time.

EZMTT method for IC_{50} and DIP rate measurement

DIP rate measurement requires freshly cultured cells. For gram-positive or negative bacteria, 10 to 80-fold dilutions in MH broth containing 1X EZMTT were added to a 96-well plate or transparent glass tubes, followed by treatment with compounds. Cell growth was measured by absorbance of 450 nm and 600 nm to generate DIP rates for each treatment. Results are representative of at least 2 independent experiments in triplicates or 3 experiments in duplicates.

Acknowledgement

We appreciate financial support from Hangzhou qinglan grant award (H1160494), Zhejiang University of Technology (414800129), The Science and Technology Planning project of Zhejiang Province (2015C33096).

Author contributions

Conception and design: B. H.R; clinical study by Q. H., L. X., C. D. EZMTT based assays by D. G., N. X., X. C., Y. Y., J. J. R.,

Competing financial interests

The authors declare no competing interests as defined by ACS or other interests that might be perceived to influence the results and /or discussion reported in this paper.

Reference:

1. World Health Organization. (2017) Global tuberculosis report 2017. World Health Organization, Geneva.
2. Makhado, N. A., Matabane, E., Faccin, M., Pincon, C., Jouet, A., Boutachkourt, F., Goeminne, L., Gaudin, C., Maphalala, G., Beckert, P., Niemann, S., Delvenne, J. -C., Delmée, M., Razwiedani, L., Nchabeleng, M., Supply, P., Jong, B. C. D., and André, E. Outbreak of multidrug-resistant tuberculosis in South Africa undetected by WHO-endorsed commercial

tests: an observational study. *Lancet Infect. Dis.Epub Oct* 17, 2018. DOI: 10.1016/S1473-3099(18)30496-1.
3. Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., and Sarkar, S. (2014) Drug resistance in cancer: an overview. *Cancers* 6, 1769-1792. DOI: 10.3390/cancers6031769.
4. Harris, L. A., Frick, P. L., Garbett, S. P., Hardeman, K. N., Paudel, B. B., Lopez, C. F., Quaranta, V., and Tyson, D. R. (2016) An unbiased metric of antiproliferative drug effect in vitro. *Nat. Methods* 13, 497-500. DOI: 10.1038/nmeth.3852.
5. Kodack, D. P., Farago, A. F., Dastur, A., Held, M. A., Dardaei, L., Friboulet, L., Flotow, F. V., Damon, L. J., Lee, D., Parks, M., Dicecca, R., Greenberg, M., Kattermann, K. E., Riley, A. K., Fintelmann, F. J., Rizzo, C., Piotrowska, Z., Shaw, A. T., Gainor, J. F., Sequist, L. V., Niederst, M. J., Engelman, J. A., and Benes, C. H. (2017) Primary patient-derived cancer cells and their potential for personalized cancer patient care. *Cell Rep.* 21, 3298-3309. DOI: 10.1016/j.celrep.2017.11.051.
6. Holohan, C., Van Schaeybroeck, S., Longley, D. B., and Johnston, P. G. (2013) Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* 13, 714-726. DOI: 10.1038/nrc3599.
7. Katsanis, G. P., Spargo, J., Ferraro, M. J., Sutton, L., and Jacoby, G. A. (1994) Detection of Klebsiella pneumoniae and Escherichia coli strains producing extended-spectrum β-lactamases. *J. Clin. Microbiol.* 32, 691-696. DOI: 10.1002/jctb.280590315.
8. Ramasamy, S., Bennet, D., and Kin, S. (2014) Drug and bioactive molecule screening based on a bioelectrical impedance cell culture platform. *Int. J. Nanomed.* 9, 5789-5809. DOI: 10.2147/ijn.s71128.
9. Eltringham, I., Pickering, J., Gough, H., Preece, C. L., and Perry, J. D. (2016) Comparison of mycobacterial growth indicator tube with culture on RGM selective agar for detection of mycobacteria in sputum samples from patients with cystic fibrosis. *J. Clin. Microbiol.* 54, 2047-2050. DOI: 10.1128/JCM.00630-16.
10. Wilson, M. L., Weinstein, M. P., Reimer, L. G., Mirrett, S., and Reller, L. B. (1992) Controlled comparison of the BacT/Alert and BACTEC 660/730 nanradiometric blood culture systems. *J. Clin. Microbiol.* 30, 323-329.
11. Caviedes, L., Delgado, J., and Gilman, R. H. (2002) Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of Mycobacterium tuberculosis. *J. Clin. Microbiol.* 40, 1873-1874. DOI: 10.1128/jcm.40.5.1873-1874.2002.
12. Zhang, W., Zhu, M., Wang, F., Cao, D., Ruan, J. J., Su, W., and Ruan, B. H. (2016) Mono-sulfonated tetrazolium salt based NAD(P)H detection reagents suitable for dehydrogenase and real-time cell viability assays. *Anal. Biochem.* 15, 33-40. DOI: 10.1016/j.ab.2016.06.026.
13. Chen, Z., Li, D., Xu, N., Fang, J., Yu, Y., Hou, W., Ruan, H., Zhu, P., Ma, R., Lu, S., Cao, D., Wu, R., Ni, M., Zhang, W., Su, W., and Ruan, B. H. Novel 1,3,4-selenadiazole-containing kidney-type glutaminase inhibitors showed improved cellular uptake and antitumor activity. *J. Med. Chem. Epub Dec* 13, 2018. DOI: 10.1021/acs.jmedchem.8b01198.
14. Savic, M., Sunita, S., Zelinskaya, N., Desai, P. M., Macmaster, R., Vinal, K., and Conn, G. L. (2015) 30S subunit-dependent activation of the *Soranguim cellulosum* Soce56 aminoglycoside resistance-conferring 16S rRNA methyltransferase Kmr. *Antimicrob. Agents Chemother.* 59, 2807-2816. DOI: 10.1128/AAC.00056-15.
15. Andrade, B., Souza, C., and Góes-Neto, A. (2013) Molecular docking between the RNA polymerase of the *Moniliophthora perniciosa* mitochondrial plasmid abd Rifampicin produces a highly stable complex. *Theor. Biol. Med. Model.* 10, 15-24. DOI: 10.1186/1742-4682-10-15.
16. Vervoort, J., Xavier, B. B., Stewardson, A., Coenen, S., Godycki-Cwirko, M., Adriaenssens, N., Kowalczyk, A., Lammens, C., Harbarth, S., Goossens, H., and Malhotra-Kumar, S. (2014) An *In Vitro* Deletion in *ribE* encoding lumazine synthasse contributes to nitrofurantoin resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* 58, 7225-7233. DOI: 10.1128/AAC.03952-14.
17. Ubukata, K., Shibasaki, Y., Yamamoto, K., Chiba, N., Hasegawa, K., Takeuchi, Y., Sunakawa, K., Inoue, M., and Konno, M. (2001) Association of amino acid substitutions in penicillin-binding protein 3 with β-Lactam resistance in β-Lactamase-negative

ampicillin-resistant *Haemophilus influenzae*. 45, 1693-1699. DOI: 10.1128/AAC.45.6.1693–1699.2001.
18. The Ministry of health the people's Republic of China. (2007) WS271-2007 Diagnostic criteria for infectious diarrhea. People's Medical Publishing House, Beijing.
19. The Ministry of health the people's Republic of China. (2008) WS287-2008 Diagnostic criteria for bacillary and amoebic dysentery. People's Medical Publishing House, Beijing.
20. The Ministry of health the people's Republic of China. (2016) WS/T80-1996 Diagnostic criteria and principles of management for food poisoning of Staphlococcus aureus. Chinese standard book publishing, Beijing.
21. The Ministry of health the people's Republic of China. (2008) WS280-2008 Diagnostic criteria for typhoid fever and paratyphoid fever. People's Medical Publishing House, Beijing.
22. Calderaro, A., Martinelli, M., Montecchini, S., Motta, F., Covan, S., Larini, S., Medici, M. C., Arcangeletti, M. C., Chezzi, C., Conto, F. D. (2016) Higher recovery rate of microorganisms from cerebrospinal fluid samples by the BACTEC culture system in comparison with agar culture. *Diagn. Micr. Infec. Dis.* 84, 281-286. DOI: 10.1016/j.diagmicrobio.2015.12.016

Table 1. Inhibition of an *E. coli* (EAEC) strain at different cell density or incubation time

	IC_{50} (μg/mL) of an *E. coli* strain				
Antibiotics[a]	**40×, 4h**	**40×, 8h**	**40×, 24h**	**400×, 8h**	**400×, 24h**
KAN	2.7±0.11	4±0.19	4±0.18	2.6±0.05	4±1.94
RIF	2±0.049	2.9±0.1	5.7±0.21	2±0.18	2.9±0.14
NIT	2.9±0.07	6.7±0.14	22.9±0.7	3.5±0.07	21±0.71
AMP	>128	>128	>128	>128	>128
PP	>256	>256	>256	3.3±0.41	>256
CAZ	5.9±0.28	2.5±0.14	2.1±0.14	<0.25	0.26
FEP	5±0.45	1.6±0.08	1.4±0.04	<0.0625	<0.0625
FOX	3.2±0.99	1.6±0.47	2.6±0.98	0.9±0.42	1.3±0.46

Kanamycin (KAN), Rifampicin (RIF), Nitrofurantoin (NIT), Ampicillin (AMP), Piperacillin (PP), Ceftazidime (CAZ), Cefepime (FEP), Cefoxitin (FOX)

Table 2. IC_{50} measurement of 5 clinically isolated bacteria strains by the EZMTT method.

Antiobiotics[b] Max. conc.	IC_{50} (μ g/mL) of various *E. coli* strains[a]									
	EAEC		EIEC		EPEC		ETEC		A	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
AMK(64μg/mL)	＜2	2.8	＜2	4.7	2.5	6.8	2.3	2.8	＜2	5.5
GEN(16μg/mL)	＜0.5	0.75	＜0.5	1.1	0.5	1.5	＜0.5	0.8	＜0.5	1.2
MNO(16μg/mL)	＜0.5	1.5	＜0.5	＜0.5	1.2	12	＜0.5	＜0.5	＜0.5	1.8
NIT(128μg/mL)	9.3	12	6.5	12	8	24	10.6	10.8	6.5	27
CSL(64μg/mL)	29.3	2.3	10.6	＜2	20	6.3	42	＜2	＞64	42.6
CZO(32μg/mL)	6	6	3.3	0.5	6.7	3	3.8	1.5	4	＞32
CTX(4μg/mL)	3.3	1	1.4	＜0.12	＞4	2	4	＜0.12	2	4
FEP(16μg/mL)	9.3	1.2	4.5	＜0. 5	5.5	2	6	＜0. 5	＜0. 5	8.6
TZP(128μg/mL)	＞128	4	64	＜4	＞128	4	4	＜4	＞128	＞128
CXM(16μg/mL)	＞16	16	＞16	4	＞16	16	＞16	3.5	＞16	＞16
CAZ(32μg/mL)	10	1	8	‹1	32	1	6	＜1	＞32	32
FOX(32μg/mL)	12	4	4.5	4.5	16	4	10.6	6	＞32	21
MEM(8μg/mL)	＜0.25	＜0.25	0.3	＜0.25	4	0.25	0.5	＜0.25	0.42	0.42
CIP(4μg/mL)	＜0.12	＜0.12	＜0.12	＜0.12	0.2	0.6	＜0.12	＜0.12	＜0.12	＜0.12
SXT(8μg/mL)	＞8	＞8	＜0.25	＜0.25	＜0.25	＜0.25	＜0.25	＜0.25	＞8	＞8

[a]Pathogenic *Escherichia coli* (EPEC), invasive *E.coli* (EIEC), toxigenic *E.coli* (ETEC), adherent *E. coli* (EAEC)

[b]Amikacin (AMK), Gentamicin (GEN), Minocycline (MNO), Nitrofurantoin (NIT),Cefoperazone/Sulbactam (CSL), Cefazolin (CZO), Cefotaxime (CTX), Cefepime (FEP), Piperacillin/tazobactam (TZP), Cefuroxime (CXM), Ceftazidime (CAZ), Cefoxitin (FOX), Meropenem (MEM), Ciprofloxacin (CIP), Sulfamethoxazole (SXT)

Table 3.Comparison the antibiotic resistant test results from clinical diagnosis and the EZMTT dye based DIP rates measurement

Strain	**EAEC**			**EIEC**			**EPEC**			**ETEC**			**A**		
	EZMTT	Assesment		EZMTT	Assesment		EZMTT	Assesment		EZMTT	Assesment		EZMTT	Assesment	
Antibiotics	Slope	EZ[a]	CL[b]	Slope	EZ[a]	CL[b]	Slope	EZ[a]	CL[b]	Slope	EZ[a]	CL[b]	Slope	EZ[a]	CL[b]
AMK(64μg/mL)	0.006	S[a]		0.003	S[a]		0.003	S[a]		0.002	S[a]		0.004	S[a]	
GEN(16μg/mL)	0.005	S[a]	S[c]	0.003	S[a]	S[c]	0.002	S[a]	S[c]	0.005	S[a]	S[c]	0.004	S[a]	S[c]
MNO(16μg/mL)	0.020	R[a]	S[d]	0.014	R[a]	S[d]	0.013	R[a]	I[d]	0.013	R[a]	S[d]	0.016	R[a]	I[d]
NIT(128μg/mL)	0.019	R[a]	S[c]	0.044	R[a]	I[c]	0.015	R[a]	S[c]	0.010	S[a]	S[c]	0.014	R[a]	I[c]
CSL(64μg/mL)	0.004	S[a]	S[d]	0.004	S[a]	S[d]	0.004	S[a]	S[d]	0.005	S[a]	S[d]	0.017	R[a]	I[d]
CZO(32μg/mL)	0.011	S[a]	S[c]	0.013	R[a]	S[c]	0.037	R[a]	S[c]	0.017	R[a]	S[c]	0.128	R[a]	R[c]
CTX(4μg/mL)	0.008	S[a]		0.006	S[a]		-0.003	S[a]		0.010	S[a]		0.062	R[a]	
FEP(16μg/mL)	0.003	S[a]	S[c]	0.007	S[a]	S[c]	0.015	R[a]	S[c]	0.009	S[a]	S[c]	-0.005	S[a]	S[c]
TZP(128μg/mL)	0.042	R[a]	S[c]	0.003	S[a]	S[c]	0.045	R[a]	S[c]	0.001	S[a]	S[c]	0.098	R[a]	S[c]
CXM(16μg/mL)	0.050	R[a]		0.009	S[a]		0.064	R[a]		0.012	S[a]		0.109	R[a]	
CAZ(32μg/mL)	0.008	S[a]	S[d]	0.004	S[a]	S[d]	0.004	S[a]	S[d]	0.004	S[a]	S[d]	0.030	R[a]	S[d]
FOX(32μg/mL)	0.005	S[a]	S[c]	0.004	S[a]	S[c]	0.003	S[a]	S[c]	0.004	S[a]	S[c]	0.012	R[a]	I[c]
MEM(8μg/mL)	0.006	S[a]	S[d]	0.012	S[a]	S[d]	0.045	R[a]	S[d]	0.013	R[a]	S[d]	0.034	R[a]	S[d]
CIP(4μg/mL)	0.005	S[a]	S[c]	0.003	S[a]	S[c]	0.005	S[a]	S[c]	0.002	S[a]	S[c]	0.114	R[a]	R[c]
SXT(8μg/mL)	0.128	R[a]	R[c]	0.008	S[a]	S[c]	0.006	S[a]	S[c]	0.003	S[a]	S[c]	0.006	S[a]	S[c]
Control(0μg/mL)	0.122			0.130			0.122			0.129			0.118		

[a] Results from EZMTT-dye based method, using 90% inhibition as the drug resistance threshold

[b] Identification based on clinical diagnostic methods

[c] Clinically used MIC (VITEK) method

[d] Clinically used KB method

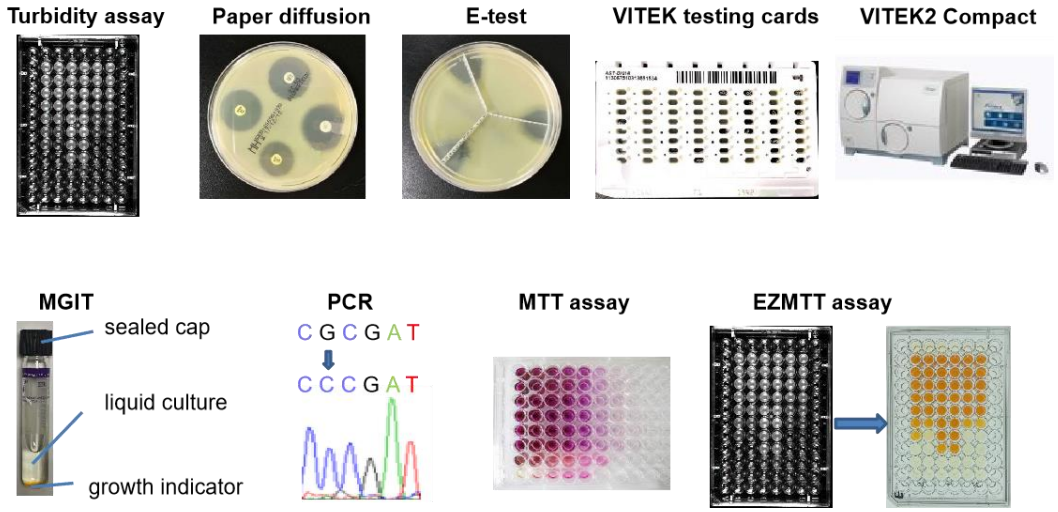


Figure 1. Main cell proliferation methods: AST methods for *E. coli* including turbidity assay, paper diffusion, broth dilution, agar dilution, E-test experiment, the automated VITEK drug sensitivity analysis system. Other available assays are oxygen detection, DNA sequencing, and MTT methods. EZMTT-dye based assay is a highly sensitive continuous assay for bacteria.

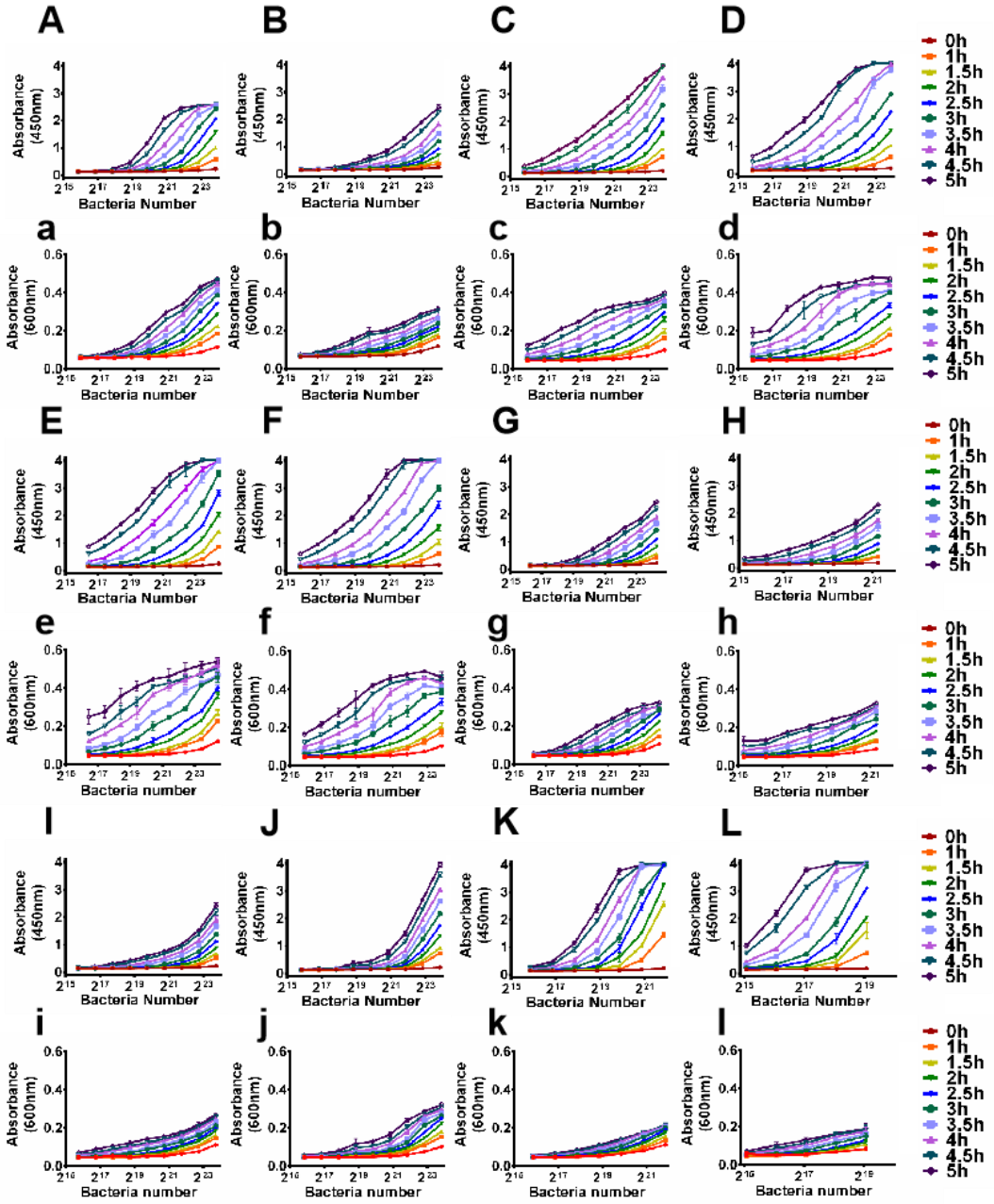
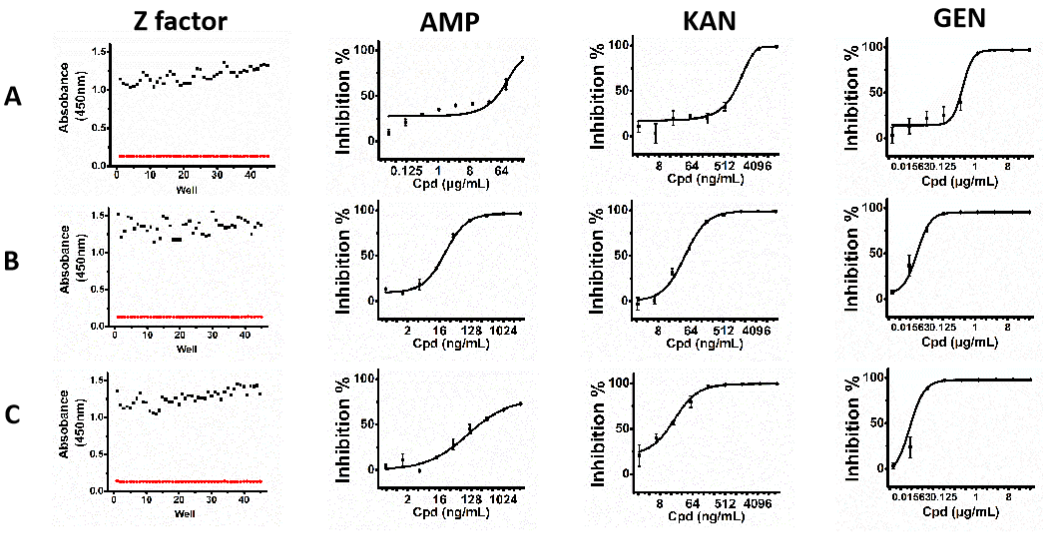


Figure 2. Growth of *E coli* strains and other infectious bacteria: Up panel (A-L): growth curve tracked by EZMTT method; Lower panel (a-l): growth curve tracked by turbidity method; The doubling times at log phase measured by the EZMTT and the turbidity methods are A) 0.80±0.11h and 0.79±0.10 h for DH5α, B) 0.88±0.00 h and 0.85±0.21 h for TransG1,C) 0.42±0.06 h and 0.42±0.08 h for EAEC(adhesive), D) 0.40±0.11 h and 0.40±0.07 h for EIEC (invasive), E) 0.43±0.09 h and 0.42±0.09 h for EPEC (pathogenic), F) 0.41±0.05h and 0.40±0.07 h for ETEC (toxigenic) *E. Coli*, G) 0.72±0.23 h and 0.74±0.06 h for *Salmonella*, H) 0.80±0.10 h and 0.71±0.08 h for *Salmonella paratyphi*, I) 1.33±0.14 h and 0.82±0.20 h for *Shigella flexneri*, J) 1.17±0.02 h and 1.30±0.27 h for *Shigella sonnei*, K) 0.50±0.07 h and 0.49±0.06 h for *Staphylococcus aureus*, L) 0.42±0.08 h and 0.47±0.18 h for *Bacillus cereus*, respectively.



D

	Full name	IC_{50} (μg/mL)			Z factor
		AMP	KAN	GEN	
A	EAEC	48±2.3	0.98±0.11	0.65±0.02	0.79
B	Staphylococcus aureus	0.02±0.01	0.05 ±0.0007	0.024±0.05	0.77
C	Bacillus cereus	0.22±0.06	0.02±0.002	0.02±0.005	0.79

Figure 3. EZMTT-dye based AST of clinically isolated (A) *E. coli*, (B) *Staphylococcus aureus* and (C) *Bacillus cereus*. (D) z factors and IC_{50} values obtained using the EZMTT dye.

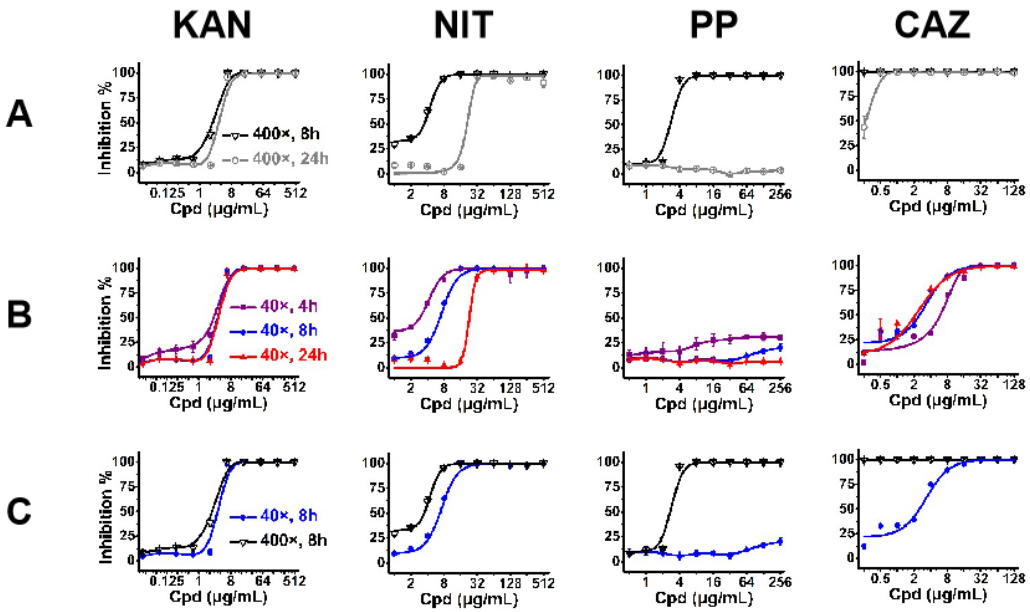


Figure 4. Changes in IC_{50} values in respond to the cell density and incubation times. Difference in IC_{50} values of EAEC strain treated with Kan, NIT, PP, CAZ to show the effects of incubation time at low (A) and high (B) cell density, and the effect of cell density (C).

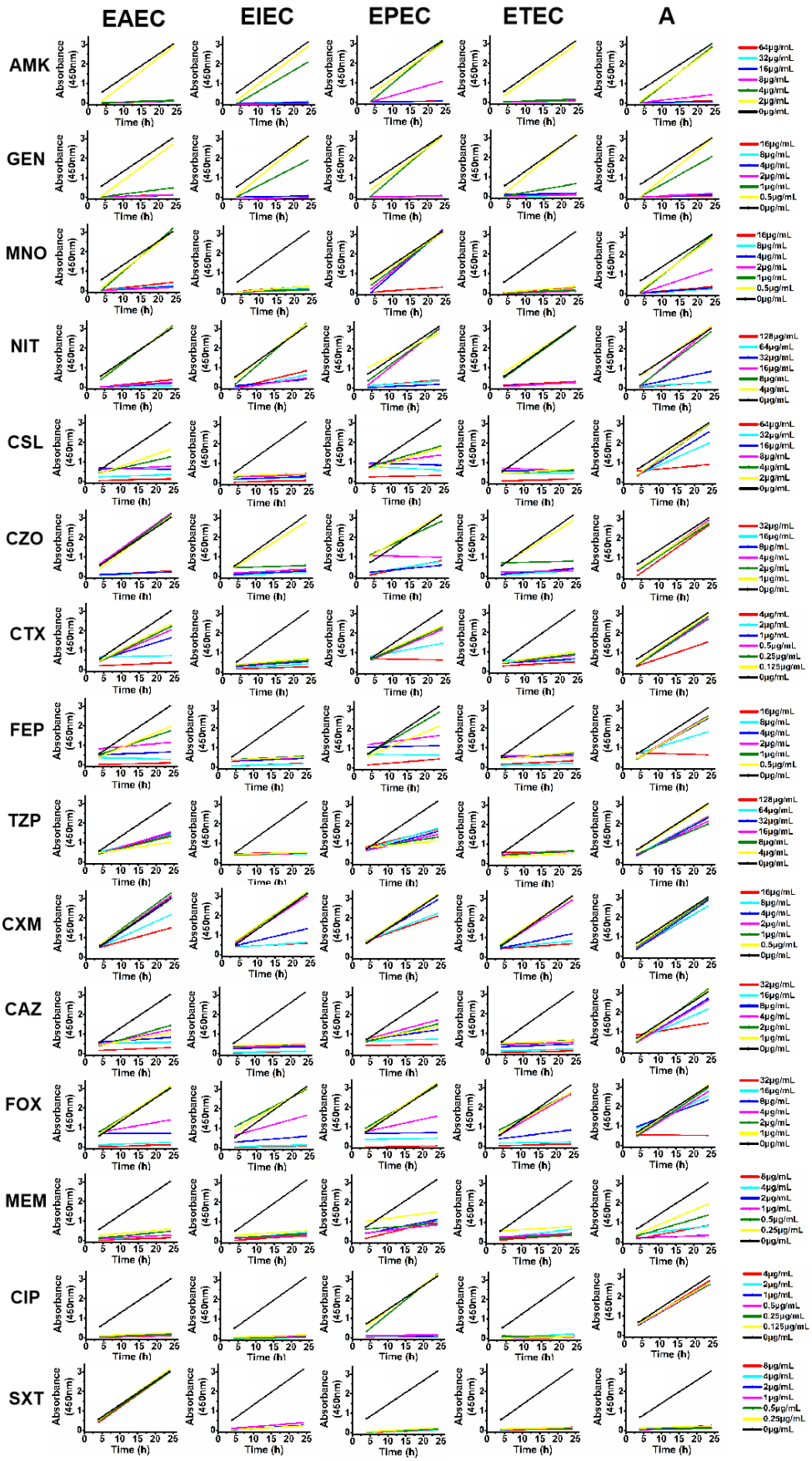


Figure 5. Time dependent growth of clinically isolated bacteria in the presence of antibiotics. The slope of cell growth at various antibiotic concentrations